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Regulation of viral expression of human immunodeficiency virus *in vitro* by an antisense phosphorothioate oligodeoxynucleotide against *rev* (*art/trs*) in chronically infected cells

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ABSTRACT In this report, we demonstrate the sequence-specific suppression of viral expression in T cells chronically infected with human immunodeficiency virus 1 (HIV-1), using antisense phosphorothioate oligodeoxynucleotides. As a target for antisense intervention, we used the HIV-1 gene *rev*, which is essential for viral replication and regulates the expression of virion proteins, in part, by affecting the splicing of the viral mRNA. A phosphorothioate oligomer complementary to the initiation sequence of HIV-1 *rev* had a significant and selective inhibitory effect on the production of several viral proteins in chronically HIV-1-infected T cells and drastically reduced the unspliced (genomic) viral mRNA transcripts, with relative sparing of smaller (spliced) transcripts. By contrast, the antisense sequence with unmodified normal phosphodiester linkages as well as phosphorothioate oligomers containing sense, random, homopolymeric sequences, or antisense sequence with N^3-methylthymidine residues did not have an inhibitory effect on viral expression. Thus, sequence specificity and nuclease resistance were critical for the anti-viral-gene regulatory effect of the antisense molecules. The altered HIV-1 mRNA profile induced by the antisense phosphorothioate oligomer suggests that the mechanism for the inhibition of viral expression is due to an interference with the regulatory gene, *rev*, by translation arrest.

The acquired immunodeficiency syndrome (AIDS) is a life-threatening disease caused by human immunodeficiency virus 1 (HIV-1) (1). While several drugs are now known to inhibit HIV replication *in vitro* and *in vivo* (2–6), no therapy now known can cure HIV infection, and the toxicities of several available drugs limit their overall efficacy in certain patients. Therefore, new antiretroviral strategies are urgently needed. In particular, future advances in the therapy of AIDS may depend on the development of therapies that can address the problem of the persistence of virus in chronically infected cells. In this context, it is worth noting that very few strategies have been proven to block expression of HIV in cells that are already infected. Rather, most antiretroviral compounds appear to act by blocking the infection of cells that are as yet uninfected (*de novo* infection) (7). In this report, we provide data showing that it is possible to inhibit the expression of HIV in chronically infected cells by exposing such cells to phosphorothioate oligodeoxynucleotides [oligo(dN)] in a complementary configuration (antisense) to the mRNA of *rev*, a critical HIV regulatory gene that is essential for efficient viral reproduction (8, 9) that regulates the expression of virion proteins.

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METHODS AND MATERIALS

Synthesis and Purification of Phosphorothioate and Unmodified Normal Oligo(dN). The phosphorothioate oligomers were synthesized by the method previously reported (6). Unmodified normal oligomers were synthesized by the standard method. All syntheses were performed with an automated DNA synthesizer (Applied Biosystems; model 380-B).

Sequences of the Target Region in the HIV Genome and Oligomers Used. We used the *rev* gene (formerly called *art/trs*) as a target for antisense intervention of viral gene expression. The *rev* gene is well conserved among HIV-1 clones. To clarify the sequence specificity, we tested an unmodified antisense oligomer of *rev* (normal phosphodiester linkages; n-α*rev*) and phosphorothioate oligomers containing *rev* sense sequence (S-sense-*rev*), *rev* antisense sequence (S-α*rev*), random sequence with the same base composition as S-α*rev* (S-random-α*rev*), 28-mer homooligomer oligo(dC)$_{28}$ (S-dC$_{28}$), *rev* antisense sequence containing four N^3-methylthymidine (*N*-MedThd) residues (S-*N*-Me-α*rev*), and an antisense sequence against the initiation site of *gag*, the gene encoding the group-specific antigen gag (S-α*gag*) (Fig. 1).

Viral Gene Expression Inhibition Assay. To test whether an agent has regulatory activity on HIV viral expression, we used chronically HIV-1-infected H9 cells, which were exposed to the virus isolate HTLV-III$_B$, kept in culture for several months or more in complete medium (RPMI 1640 supplemented with 15% fetal calf serum, 4 mM L-glutamine, 50 nM 2-mercaptoethanol, and 50 units of penicillin, and 50 μg of streptomycin per ml). Such chronically infected H9 cells (hereafter H9/III$_B$) were extensively washed to remove the previously produced viral particles from the media. After washing, H9/III$_B$ cells (1250 cells per well in a 96-well culture plate) were cultured in the presence or absence of various concentrations of oligomers in 200 μl of the culture medium above. Under these conditions, H9/III$_B$ cells could proliferate (increasing [^{3}H]thymidine uptake), and production of the virus into the culture supernatant exponentially increased for

Abbreviations: oligo(dN), oligodeoxynucleotide(s); AIDS, acquired immunodeficiency syndrome; HIV-1, human immunodeficiency virus 1; HTLV-III$_B$, particular isolate of HIV (formerly human T-lymphotropic virus type III); *N*-MedThd, N^3-methylthymidine; n-α*rev*, normal (unmodified) antisense oligo(dN) against *rev*; S-dC$_{28}$, 28-mer phosphorothioate oligo(dC); S-α*gag*, antisense phosphorothioate oligo(dN) against *gag*; S-α*rev*, antisense phosphorothioate oligo(dN) against *rev*; S-*N*-Me-α*rev*, antisense phosphorothioate oligo(dN) against *rev* containing four *N*-MedThd residues in the sequence; S-random-α*rev*, phosphorothioate random oligo(dN) with the same base composition as S-α*rev*; S-sense-*rev*, phosphorothioate sense oligo(dN) of *rev*; RIPA, radioimmunoprecipitation assay.

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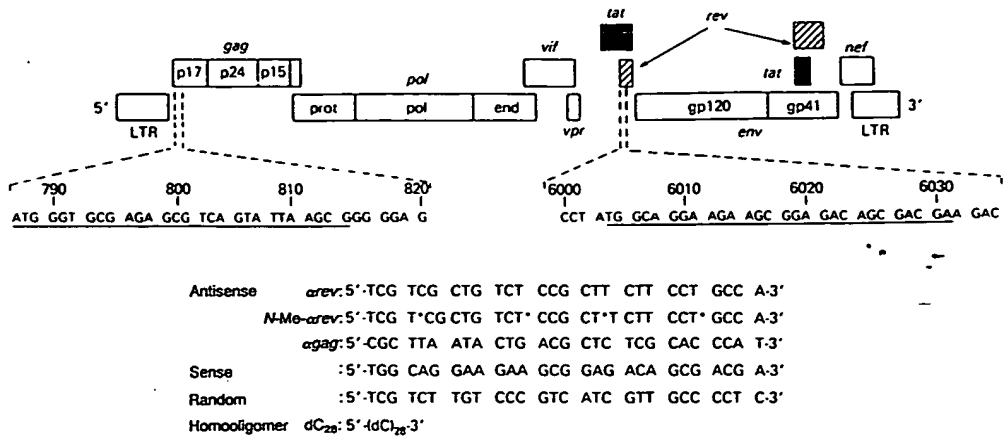

FIG. 1. DNA sequences of the initiation site of *gag* and coding exon I of *rev* in clone BH10 of HIV-1 and oligo(dN) sequences tested. Asterisks in *N*-Me-*arev* denote *N*-MedThd. The random sequence has the exact base content as antisense *rev* (*arev*) but has <70% homology with any portion in the BH10 genomic sequence as antisense or sense. In this paper, phosphorothioate analogues are denoted by "S"; normal unmodified oligo(dN)s are denoted by "n."

5 days (data not shown). After 5 days in culture, 100 μl of culture supernatants were collected and assayed for p24 gag protein by RIA (HIV p24 RIA kit, DuPont). Then, cells were pulsed for 18 hr with 0.5 μCi (1 μCi = 37 kBq) of [^{3}H]thymidine per well and harvested, and the radioactivities were counted for assessments of cytotoxicity of oligomers. For the

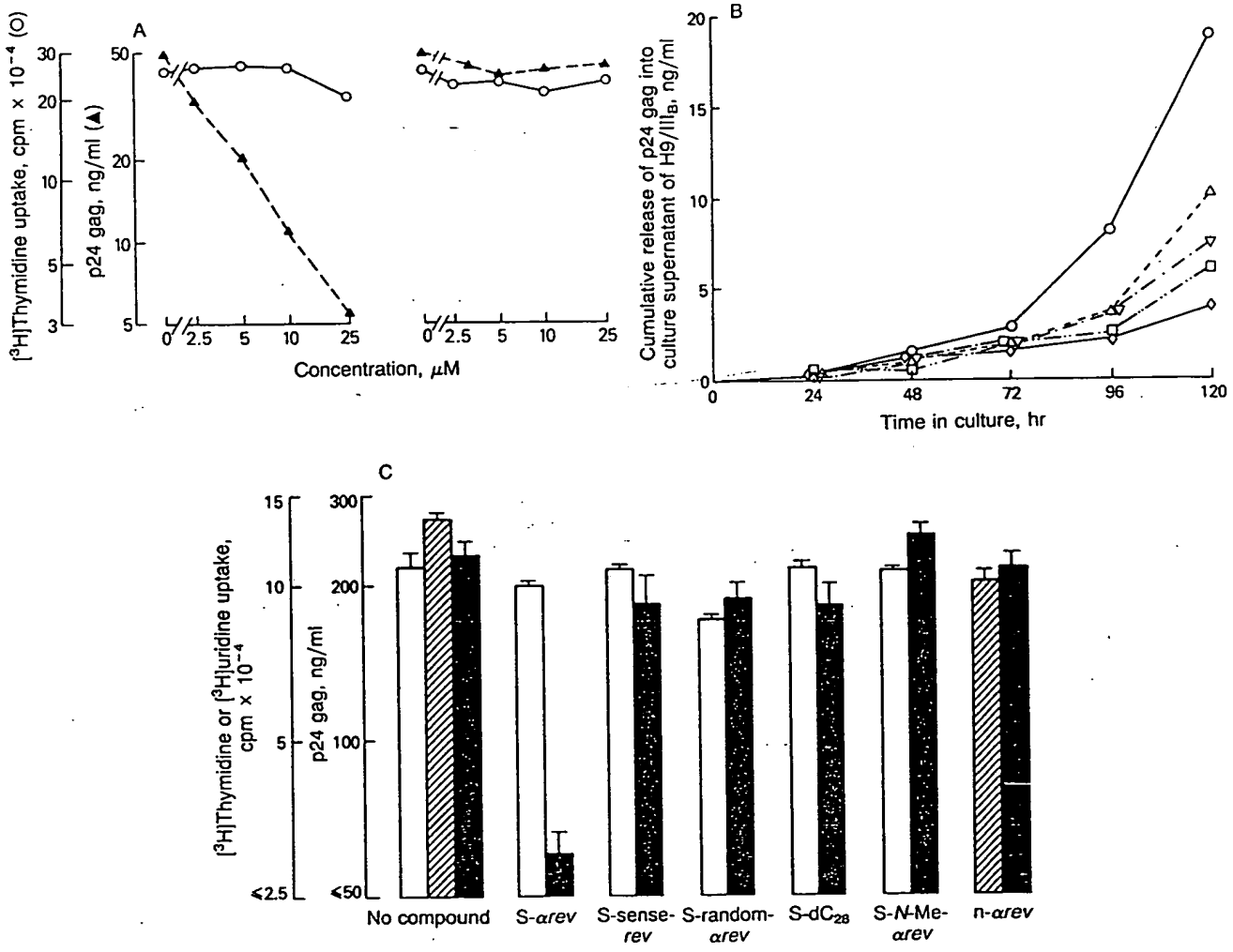

FIG. 2. (*A*) Only S-*arev* shows potent dose-dependent inhibition of the viral protein production (*Left*). The phosphorothioate homooligomer S-dC$_{28}$ showed no inhibition (*Right*). Intracellular p24 gag production per cell was similarly inhibited by S-*arev* (data not shown). (*B*) Time course of the inhibitory effect of S-*arev*. The supernatants of H9/III$_B$ in culture without (○) or with 2.5 (△), 5 (▽), 10 (□), and 25 (◇) μM S-*arev* were harvested and assayed by p24 gag RIA as in *A*. (*C*) Sequence-specific inhibition of p24 gag production. The concentrations of phosphorothioate oligomers used here were 10 μM, which did not show any toxicity to cells. Error bars represent standard deviations. □, [^{3}H]Thymidine; ▨, [^{3}H]uridine; ■, p24 gag.

normal unmodified sequence, [^{3}H]uridine was used instead of [^{3}H]thymidine because of the nuclease sensitivity of oligomers containing normal phosphodiester linkage (10). We did not make any assumption about the rapidity of any putative effects brought about by S-*arev* since this might be complex and influenced by several factors. Therefore, we performed time-course experiments. All experiments were performed in triplicate.

Radioimmunoprecipitation Assay (RIPA) of p55, p38, and p24 *gag*-Encoded Proteins and gp120 *env*-Encoded Glycoprotein. H9/III$_B$ cells (3×10^6), after 5 days in culture in the complete medium with or without 25 μM S-*arev*, were metabolically labeled with 2.5 mCi of [^{35}S]methionine and [^{35}S]cysteine (10 mCi/ml, New England Nuclear) for 4 hr (11).

RNA Analysis. Total RNA or cytoplasmic RNA from H9/III$_B$ was extracted by the guanidine thiocyanate/CsCl method (12) or the vanadyl–ribonucleoside complex method (13), respectively. RNA (10 μg) was subjected to electrophoresis on a formaldehyde/agarose gel, transferred to the Zeta probe (Bio-Rad), and hybridized with a nick-translated ^{32}P-labeled DNA [*env* region (encoding the viral envelope) of HIV-1 BH10 containing a 1.3-kilobase (kb) *Bgl* II fragment]. The RNase protection assay was performed by the method reported previously (14). A 0.6-kb *Eco*RI–*Kpn* I fragment (nucleotides 5776–6377; see Fig. 5) from the cloned HIV BH10 genome was subcloned into pGEM4 (Promega), and a uniformly labeled RNA probe was synthesized with phage T7 RNA polymerase. The human γ-actin probe was synthesized from *Hin*fI-digested pSP6-actin plasmid (a gift from Tamar Enoch; ref. 15). Two micrograms of cytoplasmic RNA and $\approx 2 \times 10^6$ cpm each of HIV and human γ-actin probe were hybridized, and RNase-protected fragments were analyzed. The concentration of oligomer used in RNA analyses was 25 μM.

RESULTS

Sequence-Specific Inhibition of Viral Expression in Chronically HIV-1-Infected T Cells. Two representative phosphorothioate oligomers, S-dC$_{28}$ and S-*arev*, were studied first. S-dC$_{28}$ has been found (5, 6) to be one of the most potent sequences tested in the cytopathic effect-inhibition assay against *de novo* infection of HIV-1 and HIV-2. However, S-dC$_{28}$ did not inhibit p24 gag production at concentrations tested in chronically HIV-1-infected H9 cells, whereas S-*arev* inhibited viral protein production ($\approx$90% at 25 μM) in a dose-dependent manner without significant toxicity (Fig. 2*A*). Fig. 2*B* depicts the time course of the sequence-specific inhibition mediated by S-*arev*, showing a lag time for the inhibitory effect. It may be worth stressing that p24 gag production occurs via the translation of the gag-pol (genomic) mRNA transcript. S-*arev* would not be expected to operate as an antisense sequence directly affecting the *gag* region *per se* but rather to function primarily against *rev* in viral regulation and expression. This lag time also may be due in part to slow internalization of the oligomer into target cells (16) and/or release of virus produced prior to exposure to the antisense. Four or five days were necessary to observe the dose-dependent inhibitory effect.

We then asked if any other sequences related to *rev* antisense sequence could inhibit viral protein production. We tested phosphorothioate oligomers S-sense-*rev*, S-*arev*, S-random-*arev*, and S-*N*-Me-*arev* as well as the unmodified n-*arev*. Fig. 2*C* shows that only the antisense sequence of the phosphorothioate analogue showed inhibition of viral p24 production without toxicity, supporting a sequence-specific regulation of viral expression of HIV in chronically infected cells. n-*arev* was sensitive to degradation by nucleases (10), and one of the degradation products, thymidine monomer, competed with [^{3}H]thymidine to depress the [^{3}H]thymidine uptake, even at nontoxic concentrations for cell growth (unpublished data). Consequently, [^{3}H]uridine was used to assess the toxicity of n-*arev*. Our failure to observe inhibition of viral protein production by n-*arev* is consistent with the view that stability of compounds is a critical factor in determining antiviral activity. N^3-Methyl substitution on the pyrimidine is known to profoundly reduce hydrogen bonding to complementary adenosine residues (19), and there was indeed no measurable duplex melting temperature (t_m) in S-*N*-Me-*arev* in contrast to the $t_m \approx 75$°C in S-*arev* against normal-sense *rev* (at pH 7, 1 M NaCl; personal communication, D. Wilson). Therefore, the inactivity of S-*N*-Me-*arev* suggests that hybridization to the complementary target mRNA is critical for inhibitory activity against the viral expression. We also tested S-*agag*, which showed significantly less inhibition than S-*arev* on viral expression (data not shown).

Inhibition of Other Viral Proteins by S-*arev*. RIPA was performed to further explore the effect of S-*arev* on the synthesis of other viral proteins. Fig. 3 shows that the production of *env*-encoded gp120 was also found to be significantly inhibited by 25 μM S-*arev*. Similar inhibition was observed for *gag*-encoded proteins including p24 and its precursors (p55 and p38) (20). These findings imply that overall viral production is inhibited rather than one particular viral protein.

Regulation of HIV-1 mRNA by S-*arev*. RNA blot-hybridization (Northern) analysis (Fig. 4) demonstrated remarkable changes in the mRNA profile of chronically infected H9 cells treated with S-*arev*. The 9.2-kb genomic mRNA of HIV, which serves also as the template for the synthesis of gag and pol, was undetectable at 5 and 28 days of culture in the continuous presence of 25 μM S-*arev*. The other partially spliced and fully spliced species of HIV-1 mRNA seemed to be comparatively spared. A time-course study of Northern blot analyses showed that a significant change of the mRNA profile occurred after 3 days of culture (data not shown), which should occur in advance of a change of protein production and, therefore, is in accord with the results of p24 gag inhibition (Fig. 2*B*). Control sequences (S-sense-*rev*, S-random-*arev*, S-*N*-Me-*arev*, S-*agag*, and S-dC$_{28}$) failed to significantly alter the mRNA profile (Fig. 4 *Lower*). In the

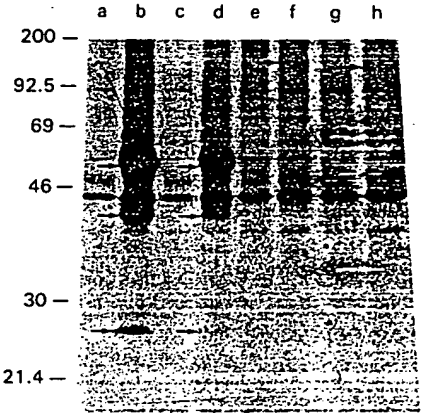

FIG. 3. RIPA of *gag*-encoded proteins and gp120 glycoprotein showing inhibition by S-*arev*. Equivalent trichloroacetic acid-precipitable radioactivity was treated with the following antibodies: 10 μl of control mouse ascites fluid generated by P3 × 63 cells (lanes a and c), 10 μl of mouse ascites fluid containing the monoclonal antibody to HIV-1 p24 (17) (lanes b and d), 5 μg of control mouse IgG (lanes e and g), and 5 μg of mouse monoclonal IgG antibody to env (18) (lanes f and h). Lanes: a, b, e, and f, no-drug control; c, d, g, and h, samples treated with 25 μM S-*arev*. The gag proteins p55, p38, and p24 indicated by arrows in lane d (S-*arev*) were greatly reduced in comparison with those in lane b (control) as was gp120 env glycoprotein, indicated by the arrow in lane h (S-*arev*), in comparison with that in lane f (control).

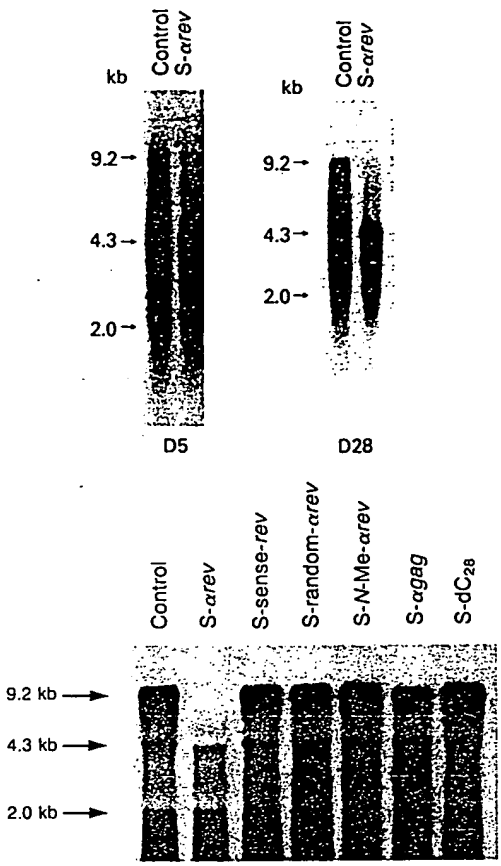

FIG. 4. (*Upper*) Northern blot analysis showing reduction of genomic mRNA of HIV-1 in the presence of S-*arev*. Total RNA from chronically HIV-1-infected H9 cells was subjected to Northern blot analysis. Note the remarkable change of mRNA profile in the presence of *S-arev*. (*Lower*) Selectivity of S-*arev* effects on the mRNA profile of HIV. Cytoplasmic RNA (10 μg) from H9/III$_B$ on day 5 in culture (no-compound control, S-*arev*, S-sense-*rev*, S-random-*arev*, S-*N*-Me-*arev*, S-*agag*, and S-dC$_{28}$) was subjected to Northern blot analysis by same method as in *Upper*. Only S-*arev* significantly altered the mRNA profile of HIV.

other set of experiments, n-*arev* failed to alter the mRNA profile (data not shown).

Fig. 5 shows a perturbation of the profile of HIV-1 mRNA by S-*arev*, supporting the findings in the Northern blot analysis. However, S-dC$_{28}$ did not alter the profile significantly. Table 1 shows the ratio of each HIV-1 mRNA band compared with the γ-actin band in the same lane and demonstrates that the unspliced genomic mRNAs of HIV-1 were the predominant species inhibited (>95% inhibition compared with no-drug control culture mRNA) in comparison with other spliced HIV mRNAs (mRNA for *tat/rev* and *env*).

DISCUSSION

The intriguing strategy of attempting to block viral replication by constructing negative-strand (antisense) oligo(dN) was first proposed by Zamecnik and Stephenson (21). However, several factors have complicated this area of research. First, oligomers with physiologic phosphodiester linkages are unstable and subject to rapid destruction by nucleases (10). Second, certain chemically modified oligomers (e.g., methylphosphonates) have inherent limitations in that they are quite insoluble and require exceedingly high concentrations for biological effects (22). Third, virtually all reported works have provided data on inhibition of *de novo* infectivity (i.e., the protection of uninfected cells) but have not provided conclusive data related to an inhibition of viral expression in

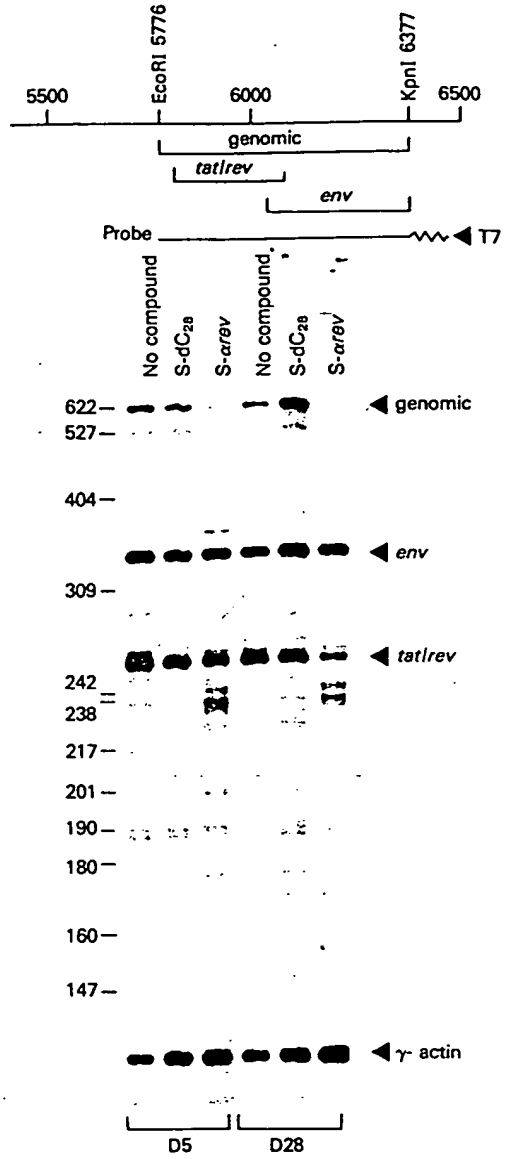

FIG. 5. RNase protection analysis of HIV-1 cytoplasmic mRNA performed with the samples on days 5 and 28. The expected sizes of fragments protected by the transcripts for *gag/pol* (genomic), *env*, and *tat/rev* are 601, 340, and 268 bp, respectively. Human γ-actin sequence served as an internal control. The numbers on the left indicate the nucleotide lengths of the *Msp* I-digested pBR322 marker. See Table 1 for the ratio of each HIV-1 mRNA band normalized by the γ-actin mRNA band.

chronically infected cells (21–27). Therefore, agents tested in such *de novo* infectivity assays might yield positive results by acting at one or more steps in the life cycle of HIV (such as binding, fusion, entry, reverse transcription, etc.) without affecting the expression of HIV genes *per se*. Complicating matters still further, it has not been possible to demonstrate sequence specificity or clear dose–response relationships in such studies (23–27).

The results provided in this report indicate that it is possible to inhibit the expression of HIV in a chronically infected cell without killing the host cell by using an antisense oligo(dN) directed against a critical regulatory gene, *rev* (8, 9). The inhibition was dose dependent and sequence specific. It may be worth stressing that the first exon of another regulatory HIV gene, *tat* (encodes the transacting activator; refs. 28 and 29), overlaps the region of the *rev* gene that serves as a target sequence for our antisense molecule (Fig. 1). Therefore, it is possible that a portion of the inhibition of viral

Table 1. Densitometric analysis of nuclease protection assay

RNA species	Day 5			Day 28		
	No compound	S-dC_{28}	S-*arev*	No compound	S-dC_{28}	S-*arev*
Genomic	0.89	0.56	0.04	0.53	0.66	0.01
env	1.98	0.96	0.77	1.09	1.22	0.81
tat/rev	2.71	1.21	0.71	1.52	1.17	0.47

Numbers in the table are normalized to the density of γ-actin message in the same lane (γ-actin = 1.00; see Fig. 5). On days 5 and 28, the ratio of unspliced mRNA to spliced mRNAs in the presence of 25 μM S-*arev* compared with that of the no-drug control culture and control culture with 25 μM S-dC_{28} was examined by scanning the autoradiography of the RNase protection assay (Fig. 4*C*). Note the drastic reduction (>95% reduction from the value of the no-drug control) of unspliced genomic HIV-1 mRNA in samples treated with 25 μM S-*arev* compared with the spliced mRNAs such as env and tat/rev.

expression observed in our studies could relate to an effect on *tat*. However, *tat* is not thought to influence viral mRNA splicing (30), suggesting that an effect on *rev* is a dominant mechanism for the effects seen in our studies.

The altered mRNA profile by S-*arev* is similar to that found in a mutant expressing low levels of *rev* (31) and is distinct from a *rev*$^-$ mutant, which expressed only the fully spliced, small mRNA transcripts (9). This suggests that inhibition of *rev* by 25 μM S-*arev* oligomer is not complete but is nevertheless adequate to partially modify the mRNA profile and significantly reduce virus production. A recent study on the role of *rev* in HIV envelope synthesis (30) suggests another possible role of *rev* in the efficient synthesis of envelope protein. The observation that *env* expression appears suppressed (Fig. 3) beyond the level of mRNA (Figs. 4 and 5 and Table 1) might support an additional role of *rev* in envelope protein production.

Combining the findings reported here and the published data on *rev* function (8, 9, 31), the antiviral activity under discussion is consistent with translation arrest of *rev*-encoded protein synthesis. The molecular details of this presumed arrest of translation will require further research and could include an enzymatic destruction of target mRNA after hybridization with the phosphorothioate analogues. It is worth noting that inhibition by antisense molecules is not a universal phenomenon. The S-*agag* did not bring about a strong inhibitory effect and did not change the mRNA profile of viral expression (Fig. 4 *Lower*).

Even after 28 days in culture, no obvious reversal of the suppressive effect on viral expression was observed (Fig. 4 *Upper*), suggesting that the phosphorothioate oligomer was durable and that no forms of the virus resistant to antisense intervention emerged. Furthermore, S-*arev* showed a comparable inhibition against the expression of HIV/RF (data not shown), which is one of the most divergent variants of HIV-1 in terms of total nucleotide sequence (32) but exhibits only one base difference in the 28 bases of the target *rev* sequence.

In AIDS and HIV-related diseases, continuous replication of the virus appears to occur and is probably essential to the pathogenesis of the disease (33). Certain phosphorothioate oligomers could inhibit both the *de novo* infection in uninfected cells (5, 6) and the expression of HIV in chronically infected cells *in vitro*, which are required to have continuous replication of HIV *in vivo*. Other studies of phosphorothioate and other classes of chemically modified antisense oligonucleotides may yield important theoretical and clinical insights into the regulation of HIV expression and replication.

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